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PRINCIPAL INVESTIGATOR: Ana M. Tari, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas
M.D. Anderson Cancer Center
Houston, TX 77030

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Ana M. Tari, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas M.D. Anderson Cancer Center
Houston, TX 77030

E-Mail: atari@mdanderson.org

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13. ABSTRACT (Maximum 200 Words)
High levels of *Wilms' Tumor 1 (WT1)* mRNA in breast tumors are linked with poor prognosis for breast cancer patients. However, the function of WT1 protein in breast cancer is not known. Recently we demonstrated that the *HER2/neu* oncogene, which is a well-known poor prognostic indicator for breast cancer patients, engages Akt to increase WT1 expression to stimulate G1 to S phase cell cycle progression and suppress apoptosis in breast cancer cells. Increased G1 to S phase cell cycle progression and decreased apoptosis are correlated with increased cyclin D1 and Bcl-2 levels. We have preliminary data indicating that Insulin-like Growth Factor-I also uses the Akt pathway to increase WT1 protein expression. We are currently investigating the role of WT1 in Insulin-like Growth Factor-I signaling. WT1 has been shown to undergo two splicing events, which result in four different isoforms. Our preliminary data indicate that all four WT1 isoforms enhance the proliferation of MCF-7 breast cancer cells, and reduce their sensitivity to tamoxifen. However, the WT1 isoforms do not appear to modulate the sensitivities of MCF-7 cells to doxorubicin and taxol. We plan to determine the mechanisms and the isoforms by which WT1 deregulates breast cancer cell proliferation and tamoxifen sensitivity.

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INTRODUCTION

The Wilms' Tumor 1 (WT1) protein and mRNA is expressed in human breast tumors and breast cancer cell lines. High levels of *WT1* mRNA have been correlated with poor prognosis for breast cancer patients. One aim of this project is to determine if the WT1 protein contributes to breast tumor progression by deregulating cell proliferation and apoptosis. The deregulation of proliferation and survival pathways has been associated with chemoresistance in many tumors. Therefore, it is hypothesized that WT1 regulates chemoresistance in breast cancer cells. WT1 has been shown to undergo two splicing events, which result in four different isoforms. These isoforms are able to bind to different DNA promoter elements and different protein partners. This project seeks to determine the mechanisms and the isoforms by which WT1 deregulates breast cancer cell proliferation and apoptosis.

BODY

Specific Aim 1: To determine whether WT1 overexpression increases the proliferation and survival of breast cancer cells in cell culture models

In the third year of funding, the Principal Investigator (PI) successfully transfected MCF-7 breast cancer cells with all four isoforms "A", "B", "C", and "D" of the *WT1* gene. Overexpression of the WT1 protein was confirmed by Western blot. Unlike our previous attempts, this time the transfection remained stable for up to at least 6 months. The CellTiter 96 Aqueous nonradioactive proliferation (MTS) assay was used to determine the proliferative rates of these transfectants. Compared to parental and vector-transfected cells, all four isoforms increase MCF-7 cell proliferation by about 130-150%. We plan to perform flow cytometry to determine in which phase(s) of the cell cycle WT1 is involved. We will then use Western blots and cDNA arrays to identify the mechanisms by which these isoforms increase breast cancer proliferation.

Specific Aim 2: To determine whether WT1 overexpression increases breast tumor growth in animal models

We have finally succeeded in obtaining stable transfectants of all four isoforms of the *WT1* gene in MCF-7 breast cancer cells. We plan to implant these transfectants into animal models and compare the growth rates of these WT1-overexpressing tumors with the parental and vector-transfected MCF-7 cells.

Specific Aim 3: To determine whether WT1 regulates chemoresistance in breast cancer cells

MTS assay was used to compare the chemosensitivity of WT1 transfectants with control cells. No difference is observed between the doxorubicin sensitivity of any of the isoforms and the control cells. Similarly, no difference is observed between the taxol sensitivity of any of the isoforms and the control cells. However, our preliminary data indicates that all four isoforms of the WT1 protein decrease the sensitivity of MCF-7 cells to tamoxifen.

Since high levels of WT1 expression had been associated with the more aggressive phenotypes of breast cancer, we believe it is important to determine what factors regulate WT1 expression. We had recently published in the journal *Oncogene* that HER2/neu increases the expression of WT1 protein to stimulate S-phase proliferation and inhibit apoptosis in breast cancer cells. Our preliminary data indicates that Insulin-like Growth Factor-I (IGF-I) uses Akt to increase WT1 expression at the post-transcriptional level. We are currently identifying the molecules downstream of Akt that are regulating WT1 expression. We are also developing WT1 siRNA molecules to inhibit WT1 expression so that we could determine the effects of WT1 inhibition on IGF-I-stimulated cell growth.

KEY ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

- Published our data that the *HER2/neu* oncogene uses the Akt pathway to increase the expression of WT1 protein. WT1 plays a vital role in mediating proliferative and anti-apoptotic functions in HER2/*neu*-overexpressing breast cancer breast cancer cells.
- Data indicates that IGF-1 uses Akt to stimulate WT1 protein expression.
- Data indicates that all four isoforms of WT1 could increase the proliferation of breast cancer cells, and decrease their sensitivity to tamoxifen.

REPORTABLE OUTCOMES:

Manuscripts

1. M. Tuna, A. Chavez-Reyes, and A. M. Tari. HER2/neu increases the expression of Wilms' Tumor 1 (WT1) protein to induce S-phase proliferation and inhibit apoptosis in breast cancer cells. Oncogene, *24:*1648-1652. (See attached)

CONCLUSIONS:

We are surprised that the four WT1 isoforms, which have been shown to bind to different partnering proteins and different DNA sequences, appear to behave quite similarly *in vitro* in the MCF-7 breast cancer cell background. Nonetheless, the project is proceeding as planned, and we will continue investigating our specific aims.

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HER2/*neu* increases the expression of Wilms' Tumor 1 (WT1) protein to stimulate S-phase proliferation and inhibit apoptosis in breast cancer cells

Musaffe Tuna[1], Arturo Chavez-Reyes[1] and Ana M Tari*[,1]

[1]*Department of Experimental Therapeutics, Unit 422, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX, USA*

High levels of the Wilms' Tumor 1 (*WT1*) protein and mRNA had been associated with aggressive phenotypes of breast tumors. Here we report that the *HER2/neu* oncogene increases WT1 expression. Approximately threefold higher levels of WT1 protein were observed in MCF-7 breast cancer cells transfected with the *HER2/neu* oncogene than in parental MCF-7 cells. Conversely, inhibition of HER2/*neu* with the anti-HER2/*neu* trastuzumab (Herceptin™) antibody decreased WT1 protein levels in HER2/*neu*-overexpressing BT-474 and SKBr3 cells. We also found that HER2/*neu* engages Akt to regulate WT1 levels since inhibition of Akt reduced WT1 levels. Decreased expression of WT1 protein led to cell cycle arrest at the G1 phase and increased apoptosis in HER2/*neu*-overexpressing cells, which is correlated with decreased cyclin D1 and Bcl-2 levels. Our data indicate that HER2/*neu* engages Akt to increase WT1 expression, and that WT1 protein plays a vital role in regulating cell cycle progression and apoptosis in HER2/*neu*-overexpressing breast cancer cells.
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Keywords: Wilms' tumor 1; HER2/*neu*; Akt; cyclin D1; Bcl-2; breast cancer

The Wilms' tumor 1 (*WT1*) gene was originally identified as a tumor suppressor gene responsible for Wilms' tumor (Call *et al.*, 1990; Haber *et al.*, 1990). In addition to germ-line mutations, somatic mutations of WT1 as well as loss of heterozygosity at the 11p13 locus harboring WT1 have been reported in sporadic Wilms' tumors. WT1 is a transcription factor that binds to CG- and TCC-rich sequences on promoters of target genes (Lee and Haber, 2001). High levels of the wild-type *WT1* mRNA had been found in leukemias (Inoue *et al.*, 1997), lung tumors (Oji *et al.*, 2002), and breast tumors (Miyoshi *et al.*, 2002). WT1 expression had been associated with the more biologically aggressive phenotypes of breast tumors, such as estrogen receptor (ER) negativity and tumors >2 cm (Silberstein *et al.*, 1997). Patients with high *WT1* mRNA levels in their breast tumors were found to have a lower 5-year disease-free survival rate than patients whose breast tumors expressed low *WT1* mRNA levels (Miyoshi *et al.*, 2002). These data strongly suggest that WT1 expression is vital to breast cancer, perhaps especially in the aggressive phenotypes. However, it is not known what factors in the aggressive breast tumors cause higher WT1 expression levels, and the functions of WT1 protein in such tumors.

Amplification of the *HER2/neu* oncogene is found in 30% of human breast cancers and is associated with poorer survival in breast cancer patients (Slamon *et al.*, 1987). HER2/*neu*-overexpressing breast tumors are of the aggressive phenotype and are likely to be ER negative (Adnane *et al.*, 1989). Thus, HER2/*neu* may be one factor that increases WT1 levels. Here we show that HER2/*neu* engages Akt to increase WT1 expression, and that WT1 protein stimulates G1/S-phase cell cycle progression and inhibits apoptosis in HER2/*neu*-overexpressing breast cancer cells, possibly via cyclin D1 and Bcl-2 proteins.

Results and discussion

HER2/neu increases WT1 protein expression in breast cancer cells

Higher levels of WT1 protein were observed in BT-474, MDA-MB-453, and SKBr3 breast cancer cell lines than in MDA-MB-231 cells (Figure 1a). All cell lines except, MDA-MB-231, express high levels of the HER2/*neu* protein (Figure 1a). Thus, we speculate that HER2/*neu* increases WT1 expression in breast cancer cells. The levels of WT1 protein were compared between MCF-7 cells transfected with the *HER2/neu* gene (MCF-7/*HER2*) and parental MCF-7 cells. Approximately three fold higher levels of WT1 protein were observed in MCF-7/*HER2* cells than in parental MCF-7 cells (Figure 1a). To further prove that HER2/*neu* increases WT1 protein expression, trastuzumab was used to inhibit HER2/*neu* function. SKBr3 and BT-474 breast cancer cell lines were incubated with 0.5 and 0.1 μM trastuzumab, respectively, for 5 days. Incubation of breast cancer cells with trastuzumab led to decreased HER2/*neu* function, as indicated by decreased

*Correspondence: AM Tari; E-mail: atari@mdanderson.org
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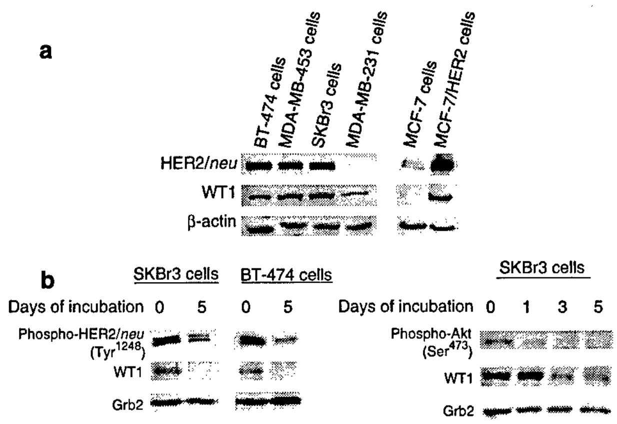

Figure 1 HER2/*neu* increases WT1 protein levels. (**a**) Breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS). The culture medium of MCF-7/HER2 cells was supplemented with 500 μg/ml G418. Western blots were performed as described (Zapata-Benavides *et al.*, 2002). Monoclonal antibodies specific for HER2/*neu*, WT1 (6F-H2), and β-actin were purchased from Oncogene (Cambridge, MA, USA), DAKO (Carpinteria, CA, USA), and Sigma (St Louis, MO, USA), respectively. Protein bands were visualized by enhanced chemiluminescence (Kirkegaar & Perry Laboratories, Gaithersburg, MD, USA). Images were scanned and quantified by an Alpha Innotech densitometer using the Alpha Imager application program (San Leandro, CA, USA). Levels of WT1 were normalized to those of β-actin. (**b**) SKBr3 and BT-474 cells were plated at 0.75 and 1.00×10^5 cells/well, respectively, in six-well plates. After overnight attachment, trastuzumab (kindly provided by Genentech, San Francisco, CA, USA), was added to SKBr3 and BT-474 cells at final concentrations of 0.5 and 0.1 μM, respectively. Western blots were performed. Antibodies specific for phospho-HER2/*neu* (Tyr^{1248}) and phospho-Akt (Ser^{473}) were purchased from Cell Signaling Technology (Beverley, MA, USA). Grb2 protein was used as a loading control

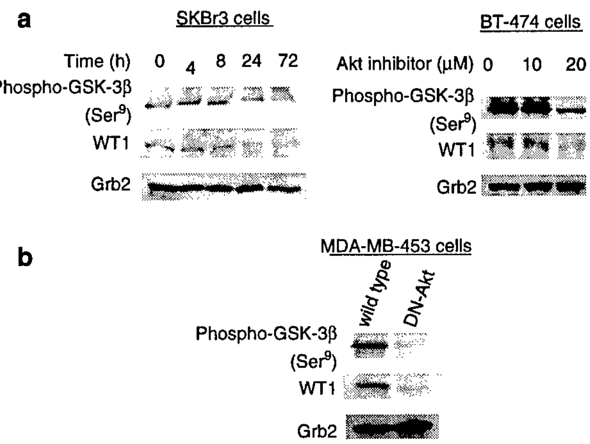

Figure 2 Inhibition of Akt activity leads to reduced WT1 protein levels in HER2/*neu*-overexpressing breast cancer cells. The Akt inhibitor (IL-6-hydroxymethyl-chiro-inositol-2^{R}-20-methyl-3-*O*-ocadecylcarbomate) and the phospho-GSK-3β (Ser^9) antibody were purchased from Calbiochem (San Diego, CA, USA) and Cell Signaling Technology, respectively. (**a**) SKBr3 and BT-474 cells were plated at 0.75 and 1.00×10^5 cells/well, respectively, in six-well plates in DMEM/F12 medium containing 5% FBS. SKBr3 cells were treated with 10 μM Akt inhibitor for 0, 4, 8, 24, and 72 h, while BT-474 cells were treated with 0, 10 or 20 μM Akt inhibitor for 72 h. Levels of WT1 and phospho-GSK-3β were determined. (**b**) Western blots were performed to determine the levels of WT1 protein in MDA-MB-453 wild-type and MDA-MB-453/DN-Akt cells. Grb2 was used as loading control

phosphorylation of the Tyr^{1248} residue in the HER2/*neu* protein and decreased phosphorylation of the Ser^{473} residue in the Akt protein (Figure 1b). Blocking *HER2/neu* led to WT1 protein levels decreased by 51 and 43% in SKBr3 and BT-474 cells, respectively (Figure 1b). Reduced WT1 protein expression in SKBr3 cells was also observed after 3 days incubation with the trastuzumab antibody (Figure 1b). These data indicate that HER2/*neu* increases WT1 protein levels.

*HER2/*neu *engages Akt to increase WT1 protein expression*

Akt is an important downstream signaling protein of HER2/*neu* (Zhou *et al.*, 2000; Lenferink *et al.*, 2001). To determine whether HER2/*neu* engages Akt to regulate WT1 expression, SKBr3 and BT-474 cells were incubated with an Akt inhibitor. As expected, the Akt inhibitor decreased the phosphorylation of the Ser^9 residue of GSK-3β, a downstream protein of Akt. The Akt inhibitor decreased WT1 protein expression in SKBr3 and BT-474 cells (Figure 2a). By 72 h, the Akt inhibitor decreased WT1 levels by 45 and 61% in SKBr3 and BT-474 cells, respectively. To further confirm that Akt is involved in the regulation of WT1 expression, the levels of WT1 protein were compared between MDA-MB-453 wild-type cells and MDA-MB-453 cells stably transfected with the dominant-negative Akt mutant cDNA (DN-Akt) (Zhou *et al.*, 2000). Approximately two fold higher levels of WT1 protein were observed in MDA-MB-453 wild-type cells than in DN-Akt cells (Figure 2b). These data indicate that Akt is vital for the increased WT1 expression in HER2/*neu*-overexpressing cells.

*Downregulation of WT1 protein expression induces cell cycle arrest and apoptosis in HER2/*neu*-overexpressing breast cancer cells*

Previously, we demonstrated that WT1 protein is vital for the growth of BT-474 and SKBr3 cells, since downregulation of WT1 protein expression by liposome-incorporated WT1 (L-WT1) antisense oligos led to growth inhibition in both cell lines (Zapata-Benavides *et al.*, 2002). However, it is not known in which phase of the cell cycle WT1 is involved. BT-474 cells were incubated with L-WT1 antisense oligos and liposomal control (L-control) oligos for 5 days. Flow-cytometric analysis was used to determine which phase of the cell cycle WT1 is involved. In BT-474 cells, L-WT1 antisense oligos increased the percentage of cells in the G1 phase by 16%, and decreased the percentage of cells in the S phase by 15%. L-WT1 antisense oligos also increased the percentage of BT-474 apoptotic cells, as indicated by the sub-G1 phase, from 2.2 to 37.8% (Figure 3). However, L-WT1 antisense oligos did not affect the

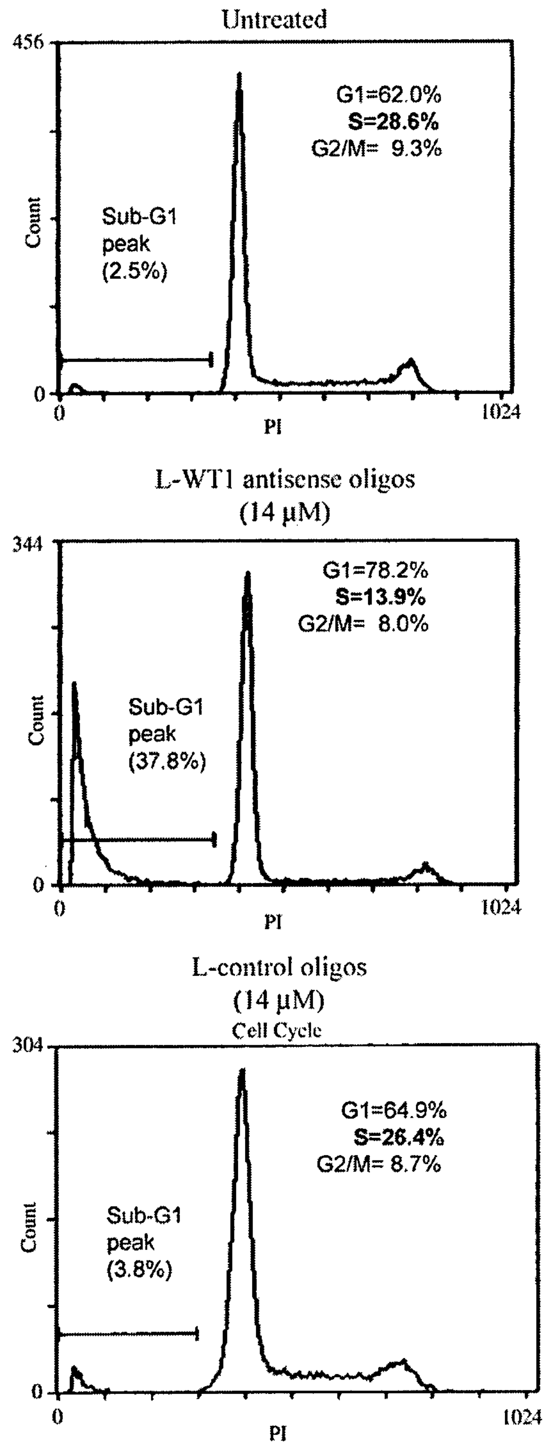

Figure 3 WT1 protein mediates proliferative and antiapoptotic functions in BT-474 breast cancer cells. Liposomal oligos, with the following sequences: WT1 antisense, 5′-GTC GGA GCC CAT TTG CTG-3′ and control oligo, 5′-TCG CGA CTG GAT CCT GCC CG-3′, were prepared as described (Zapata-Benavides et al., 2002). BT-474 cells, plated at 0.75×10^5 cells/well in six-well plates, were treated with 14 μM L-WT1 antisense or L-control oligos for 5 days. Cell cycle and apoptosis were evaluated by flow-cytometric analysis of propidium iodide staining using a Coulter Epics Profile 488 laser as described by Simeone et al. (2004)

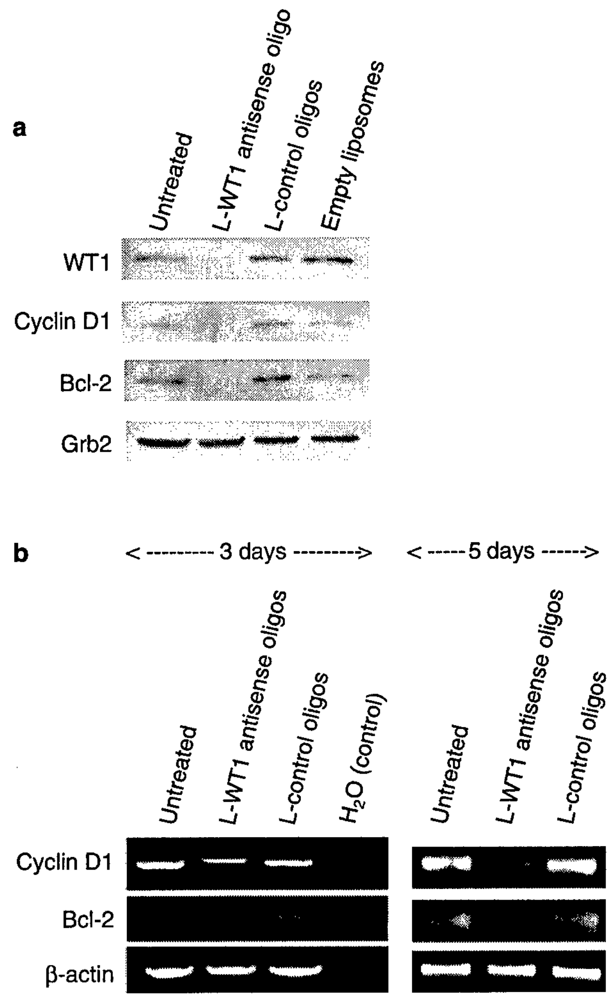

Figure 4 WT1 protein regulates cyclin D1 and Bcl-2 expression in BT-474 cells. (**a**) BT-474 cells were incubated with 14 μM liposomal oligos or empty liposomes for 5 days. Western blot was used to determine the effects of WT1 downregulation on the levels of cyclin D1 and Bcl-2 proteins. Grb2 protein was used as a loading control. (**b**) BT-474 cells were incubated with 14 μM liposomal oligos for 3 or 5 days. RT–PCR was used to determine the effects of WT1 downregulation on *cyclin D1* and *bcl-2* mRNA levels. β-Actin was used as loading control. Total RNA was isolated by the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). cDNA was created with Superscript II (Invitrogen). All PCR reactions were carried out with 5 μl cDNA, 0.2 mM dNTPs, 2.5 μM of each primer, 10 mM Tris–HCl (pH 8.4, 50 mM KCl, 0.01% gelatin, 2.0 mM $MgCl_2$), and 2.5 U Taq DNA polymerase. The sequences of the cyclin D1 primers were: forward, 5′-CTG GAG CCC GTG AAA AGA GC-3′ and reverse, 5′-CTG GAG GAA GCG TGT GAG G-3′. The sequences of the Bcl-2 primers were: forward, 5′-TGC ACC TGA CGC CCT TCA C-3′ and reverse, 5′-AGA CAG CCA GGA GAA ATC AAA CAG-3′. The sequences of the β-actin primers were: forward, 5′-GTC ACC AAC TGG GAC GAC ATG-3′ and reverse, 5′-GAC AGC ACT GTG TTG GCG TAC-3′. The PCR conditions for cyclin D1 were: 94°C for 3 min, five cycles of 94°C for 30 s and 72°C for 45 s, 25 cycles of 94°C for 30 s, 56°C for 1 min and 72°C for 2 min, followed by 7 min at 72°C. Identical PCR conditions were used for Bcl-2, except that 30 cycles were used. The PCR conditions for β-actin were 94°C for 5 min, and 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 45 s, followed by 7 min at 72°C. PCR products, subjected to electrophoresis on 1% agarose gels, were visualized with ethidium bromide and photographed under UV transillumination

G2/M phase. Under the same conditions, L-control oligos did not affect the cell cycle distribution (Figure 3). Similar effects were observed in SKBr3 cells. L-WT1 antisense oligos increased the percentage of SKBr3 cells in the G1 phase by 10%, but did not affect the G2/M phase (data not shown). L-WT1 antisense oligos also increased the percentage of SKBr3 cells undergoing apoptosis from 5.9 to 10.7% (data not shown). These data indicate that WT1 stimulates G1- to S-phase cell cycle progression and inhibits apoptosis in HER2/*neu*-overexpressing breast cancer cells.

WT1 protein regulates cyclin D1 and Bcl-2 *expression at the transcription level*

We then determined which cell cycling and apoptotic proteins may be regulated by WT1. Cyclin D1 is a downstream target of HER2/*neu*, and is essential for *HER2/neu* to induce mammary tumorigenesis in transgenic mice (Lee *et al.*, 2000; Lenferink *et al.*, 2001). The expression of *bcl-2* and *WT1* mRNA is significantly correlated in leukemic blasts and is associated with reduced survival in patients with acute myelogenous leukemia (Karakas *et al.*, 2002). One possible mechanism by which WT1 prevents cells from undergoing apoptosis is by upregulating the antiapoptotic *bcl-2* gene. Western blot was performed on untreated BT-474 cells and those treated with liposomal oligos and empty liposomes. L-WT1 antisense oligos inhibited WT1 protein expression by 50% (Figure 4a), whereas L-control oligos and empty liposomes did not affect WT1 protein expression (Figure 4a). Downregulation of WT1 protein expression led to decreased cyclin D1 and Bcl-2 protein levels (Figure 4a).

WT1 has been shown to increase *bcl-2* transcription (Mayo *et al.*, 1999). We speculate that WT1 regulates *cyclin D1* transcriptionally because the *cyclin D1* promoter contains CG-rich elements that are potential WT1 consensus sequences. To determine whether WT1 regulates *bcl-2* and *cyclin D1* transcriptionally, treated and untreated BT-474 cells were subjected to RT–PCR. L-control oligos and empty liposomes did not have any effects on *bcl-2* and *cyclin D1* levels (Figure 4b). Downregulation of WT1 protein expression by L-WT1 antisense oligos led to decreased *bcl-2* and *cyclin D1* mRNA levels (Figure 4b).

Our finding that WT1 upregulates the transcription of the *bcl-2* gene agrees with that of Mayo *et al.* (1999). However, the *bcl-2* promoter has also been shown to be negatively regulated by WT1 (Hewitt *et al.*, 1995; Cheema *et al.*, 2003). Differences in cell types, status of WT1 isoforms, and the interaction of WT1 protein with other proteins may explain the differences between our results and those of Hewitt *et al.* (1995) and Cheema *et al.* (2003).

Our finding is the first to show that WT1 increases *cyclin D1* mRNA levels, and that, in addition to Akt (Lenferink *et al.*, 2001), WT1 is another mechanism by which HER2/*neu* stimulates cyclin D1 expression. The activation of Akt leads to increased cell growth and survival by phosphorylating and inactivating some of the downstream targets such as BAD (Datta *et al.*, 1996), pro-caspase 9 (Cardone *et al.*, 1998), GSK3-β (Gold *et al.*, 1999; Takata *et al.*, 1999), and forkhead transcription factors (Brunet *et al.*, 1999). Akt activates downstream proteins by phosphorylating them at the consensus sequence site (RXRXXS). Since an Akt consensus site cannot be found within the WT1 sequence, Akt is likely to increase WT1 levels via other downstream factors.

Previously, we showed that 17β-estradiol increased WT1 protein expression in ER-positive breast cancer cells (Zapata-Benavides *et al.*, 2002). Here, we report that HER2/*neu* increases WT1 levels, and that WT1 has a key role in mediating proliferative and antiapoptotic effects in HER2/*neu*-overexpressing cells, possibly by regulating the transcription of *cyclin D1* and *bcl-2*. Our data support earlier observations that WT1 may play a vital role in the aggressive phenotypes of breast cancer cells (Miyoshi *et al.*, 2002). WT1 may be used as a novel therapeutic target in HER2/*neu*-overexpressing breast cancer.

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